ELECTRON MICROSCOPIC EXAMINATION OF AUTOPHAGY AND CYTOPLASMIC DEGRADATION INDUCED BY CADMIUM CHLORIDE AND HYPEROSMOTIC SUCROSE IN EXOCRINE PANCREATIC CELLS OF MICE

by

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Introduction

Numerous data have become available in recent years on the degradation of various origin of the cytoplasm. Examining the effects of metabolic inhibitors on liver and exocrine pancreatic cells, Hruban and coworkers found that these substances brought about a cytoplasmic degradation of focal character (Hruban, Spargo, Swift, Wissler, Kleinfeld, 1963; Swift and Hruban, 1964); a great number of autophagic vacuoles (De Duve and Wattiaux, 1966) develop in the cell. A similar effect is exerted by hyperosmotic sucrose solution in the cells of the proximal tubuli of the kidney (Trump and Janigan, 1962), in liver parenchymal cells (Brewer and Heath, 1963; Brewer and Heath, 1964; Wattiaux, Wattiaux-De-Coninck, Ruttgeerts and Tulkens, 1964), in the epithelial cells of the seminal vesicle (Kovács, 1968) and in exocrine pancreatic cells (Réz and Kovács, 1967). In exocrine cells of the pancreas autophagy was observed, further, upon the effect of protein-deficient nutrition (Weissblum, Hermann and Fitzgerald, 1961), of diethanolamine and triparanol (Hruban, Swift and Slesers, 1965/b), of azaserine (Hruban, Swift and Slesers, 1965/a), of 3-thienyl-DL-alanine (Swift and Hruban, 1964), of 3-furyl-DL-alanine (Hruban, Swift, Dunn and Lewis, 1965), of neutral red (Alousi, Morgan and Stenger, 1967; Réz and Kovács, 1967, 1971) and of cadmium II ions (Réz and Kovács, 1969).

Autophagic vacuoles, as bodies containing substance separated from other parts of the cytoplasm by a membrane, are parts of the vacuolar system. De Duve's extra cellular space (De Duve, 1969) takes place in their interior. The most generally accepted conception on the origin of their membrane is that it derives from pre-formed membranes, thus from the smooth-surfaced endoplasmic reticulum of the GERL complex (Novikoff, Essner and Quintana, 1964), from the

rough-surfaced or smooth-surfaced ER (Ericsson, 1965; Ericsson and Glinsman, 1966; Ericsson, 1969) or from the Golgi apparatus (Cohn and Fedorko, 1969; Fedorko, Hirsch and Cohn, 1969; Fedorko, Hirsch and Cohn, 1968; Fedorko, 1968; Frank and Christensen, 1968). According to Maunsbach's findings (Maunsbach, 1969), in the epithelial cells of the proximal tubule of the kidney, autophagic vacuoles are delimited by two or more membranes originating probably from the smooth-surfaced endoplasmic reticulum or from the Golgi apparatus. The thickness of these membranes proved to be 65 Å each, which equals that of the ER membrane. In the case of autophagic vacuoles delimited by a single membrane the membrane shows 90 Å thickness. This may be a morphological evidence for the fact that the preformed thin membrane responsible for the encapsulation of the parts of the cytoplasm transforms into a thick one by fusion and becomes similar to the thick membranes of the vacuolar system. Consequently, the delimiting membrane may originate from various membranes, depending on the type of cells.

Generally, the digestion of the content of the vacuoles is belived to be slow since in their initial forms, the presence of acid phosphatase could not be demonstrated. The opinion that the enzymes are transported there by primary or secondary lysosomes (Arstila and Trump, 1968; Ericsson, 1968; Ericsson and Glinsman, 1966; Ericsson, 1969; Fedorko, Hirsch and Cohn, 1969; Fedorko, Hirsch and Cohn, 1968; Fedorko, 1968; Glinsman and Ericsson, 1966), or that they derive from the Golgi cisterns effecting the segregation (Frank and Christensen, 1968) is general.

According to earlier findings of the authors, there are several types of autophagic vacuoles to be distinguished as to the morphology of the segregated substance (Kovács and Réz, 1970; Réz and Kovács 1971). The various types of morphology probably represent various forms of autophagic degradation.

In order to obtain further data on the origin of the limiting membranes, acid hydrolase content, and morphological types of autophagic vacuoles, autophagy was induced in mouse exocrine pancreatic cells by intraperitoneal injections of hyperosmotic sucrose and/or cadmium II chloride solutions.

Material and methods

Thirtyfive female albino mice weighing 25—30 g each were used. Group I of the animals was given 1 ml 15% sucrose solution intraperitoneally then, upon periods of 10, 30, 60, 120 and 180 minutes, they were decapitated; 5 animals at each time. Group II of the mice (10 animals) was given 0.2 ml of aqueous cadmium-II chloride ($CdCl_2$) solution of 0.8 mg/ml concentration intraperitoneally, then after 2 hours they were decapitated. The pancreatic pieces of the animals were fixed for 2 hours partly

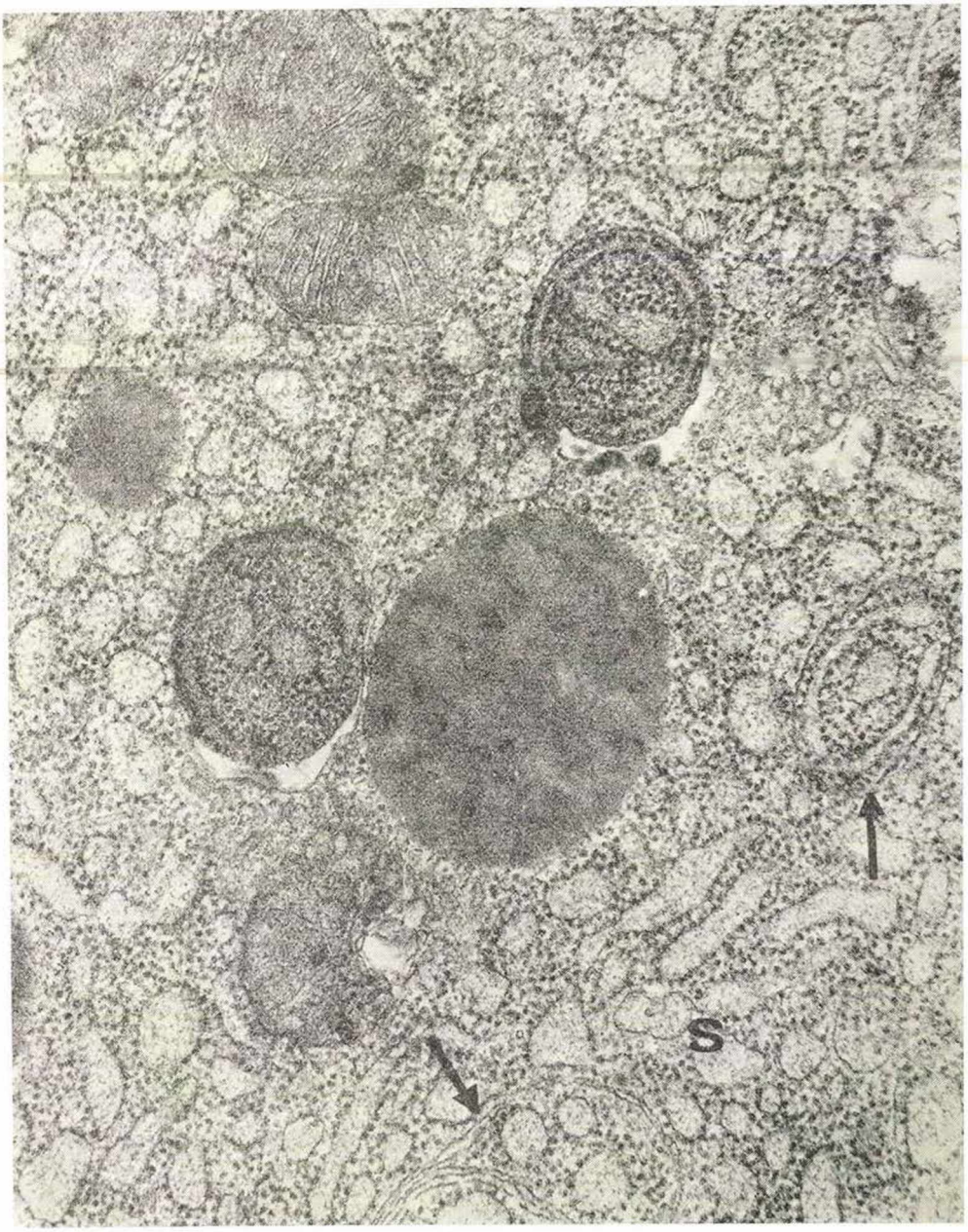

Fig. 1. Autophagic vacuoles 2 hours upon cadmium treatment. A young form *(arrow)* and type of increased osmiophilia are shown. Smooth-surfaced membranes (s) and, near the vacuoles, vesicles are present. (x 40300)

in 1% osmium tetroxide dissolved in 0.125 M neutral phosphate buffer, partly in a glutaric aldehyde solution (Anderson, 1970; Vadász 1966), purified in ion-exchanging synthetic resin (Varion), which was followed by a post-fixation for 1 hour in a 1% osmium tetroxide solution, then embedded in Araldit. The ultrathin sections were contrasted by means of uranyl acetate and lead citrate. To demonstrate the activity of acid phosphatase, 10 μ thick freezed sections were prepared from the samples fixed in glutaric aldehyde, and incubated for 20—40 minutes in a modified Gömöri medium (Geyer, 1969), then embedded in Araldit. A UEMV—100 B type electron microscope was used for the examinations.

Results

The authors did not find any principal differences between the effects of sucrose and cadmium chloride solutions. Both treatments caused focal changes of the cytoplasm; morphologically the effect of the two substances cannot be distinguished. Thirty minutes after the treatments a marked autophagy can be observed in the cells. The number and size of autophagic foci reached a maximum 1—2 hours later. The changes are the following:

Fig. 2. 15% sucrose solution, 3 hours. Autophagic vacuoles containing highly dense material. (x 38500)

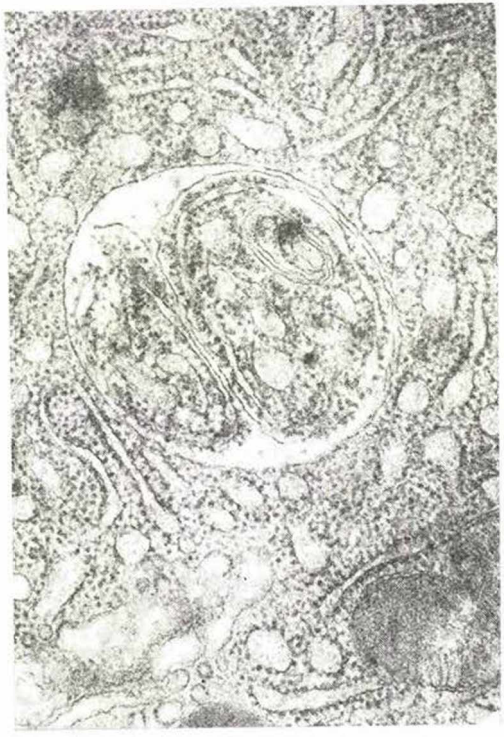

Fig. 3. 15% sucrose solution, 30 minutes. Autophagic vacuole containing fragmented cisternae, in which increase in density cannot be observed. (x 31300)

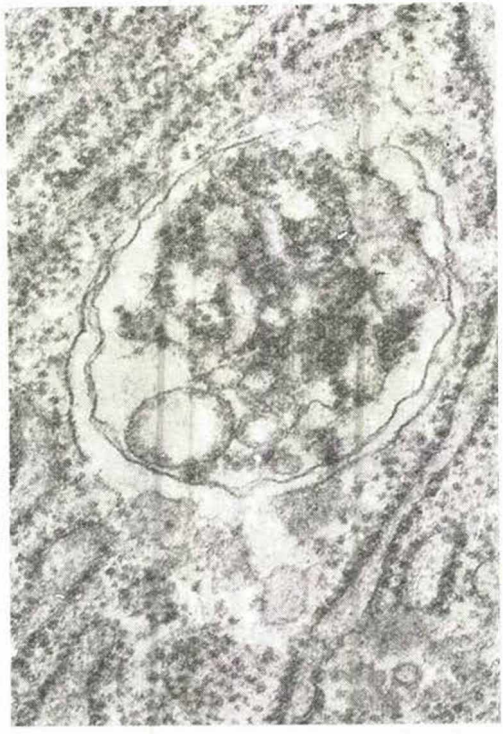

Fig. 4. Cadmium chloride, 2 hours. Autophagic vacuole containing degrading cisternae and vesicles. (x 56000)

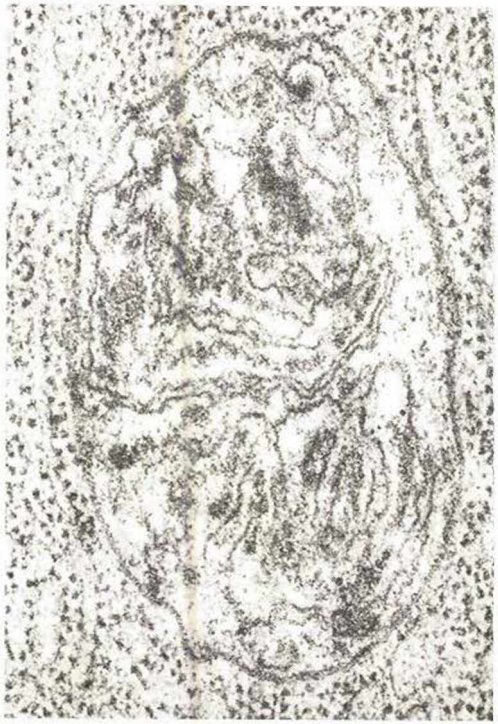

Fig. 5. Autophagic vacuole in a cell treated with cadmium chloride. Pairs of membranes and filamentous-granular substance can be seen in the interior. (x 40000)

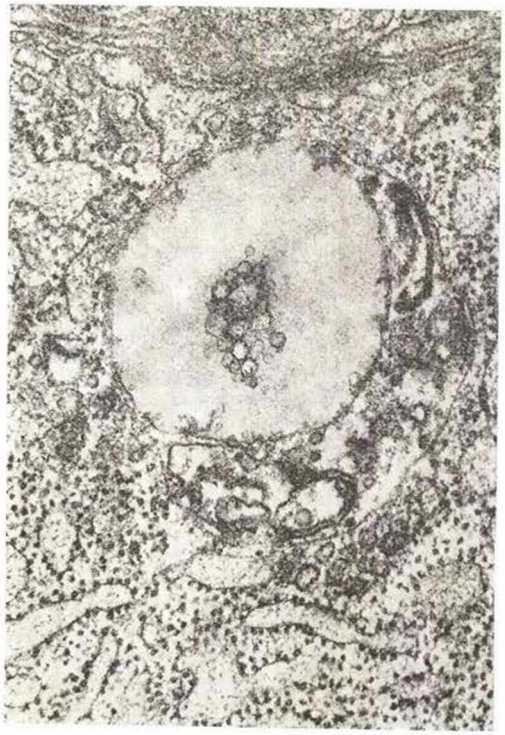

Fig. 6. 15% sucrose solution, 3 hours. A vacuole containing vesicles, membranes is seen in the vicinity of the Golgi apparatus. (x 40 300)

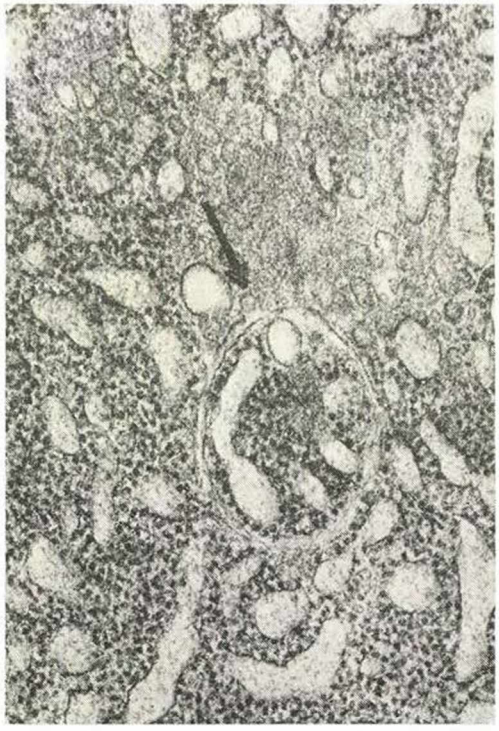

Fig. 7. Cadmium chloride, 2 hours. Autophagic vacuole in the immediate vicinity of the Golgi apparatus. A Golgi vesicle is fusing *(arrow)* into the outer membrane. (x 55000)

Fig. 8. 15% sucrose solution, 30 minutes. Young autophagic vacuole delimited by two pairs of membranes. The inner one is smooth-surfaced and in part already fused. It is surrounded by a rough-surfaced ER cistern, from the surface from which the ribosomes are missing in part. The two ends of the rough surfaced cistern fuse at the spot marked with an arrow, and it encircles the vacuole. (x 28000)

1. Great numbers of autophagic vacuoles, containing parts of rough-surfaced endoplasmic reticulum appear in the cytoplasm. Those containing undegraded material delimited by a pair of unfused membranes can be considered young and newly formed. (Figures 8 and 9). The older autophagic vacuoles are delimited by a partly or fully fused pair of membranes, the encapsuled substance shows signs of decomposition. The older vacuoles can be ranged with two main types. Some of them

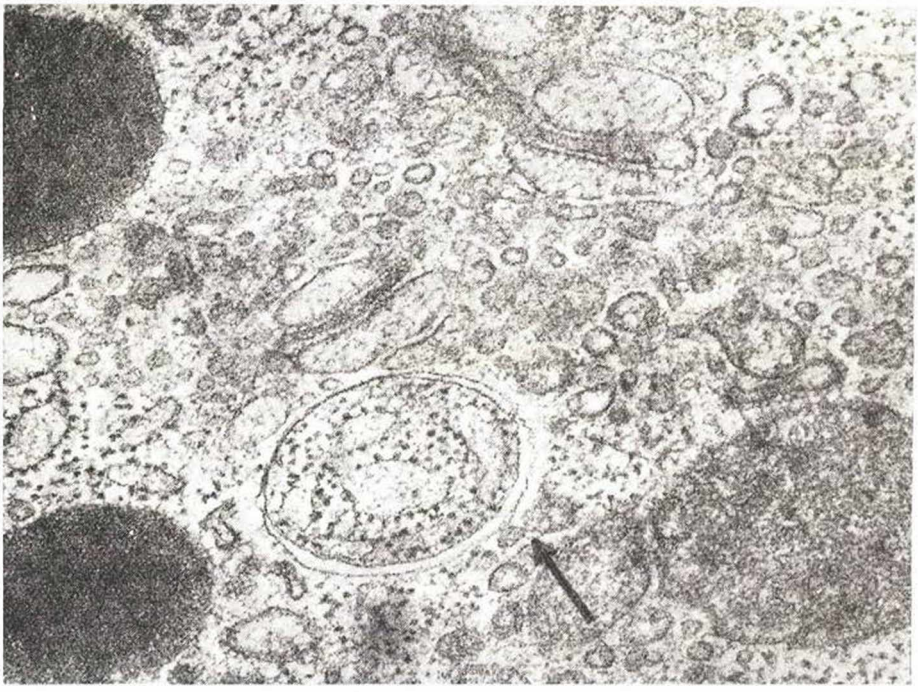

Fig. 9. 15% sucrose, 30 minutes. Young autophagic vacuole with partly fused membrane in the Golgi area. External member of delimiting membrane is in connection with a Golgi vesicle (arrow). (x 36200)

(Figure 1) contain well recognizable ER cisternae. The ribosomes are placed regularly on the surface of the cisternae. However, the density of the content of the vacuoles significantly increases, as compared with that of the surrounding cytoplasm. According to the increasing osmiophilia, a progressive series can be set up, at the end of which there are the forms similar to the two autophagic vacuoles containing highly dense substance as presented in Figure 2. Here the cisternae of the endoplasmic reticulum are difficult to verify. In most cases the vacuole of this type is filled with dense material ordered in lines parallel with membranes. Also the vacuole shown in Figure 5 might belong with this decomposition series. It contains paralelly arranged membrane fragments, with granulous dense

material in between. Relying on the arrangement of the membranes, one can suppose that they derive from degraded ER cisternae. Of the other type of autophagic vacuoles it is characteristic that the material included in them shows signs of decomposition, however, without an increase in density. Decomposition is indicated by the fact, that the encapsuled cisternae are transformed into minor vesicles, the surfaces of which are partly or entirely free of ribosomes (Figures 3 and 4). Relying on the structure of the degrading matter, the existence of a progressive series

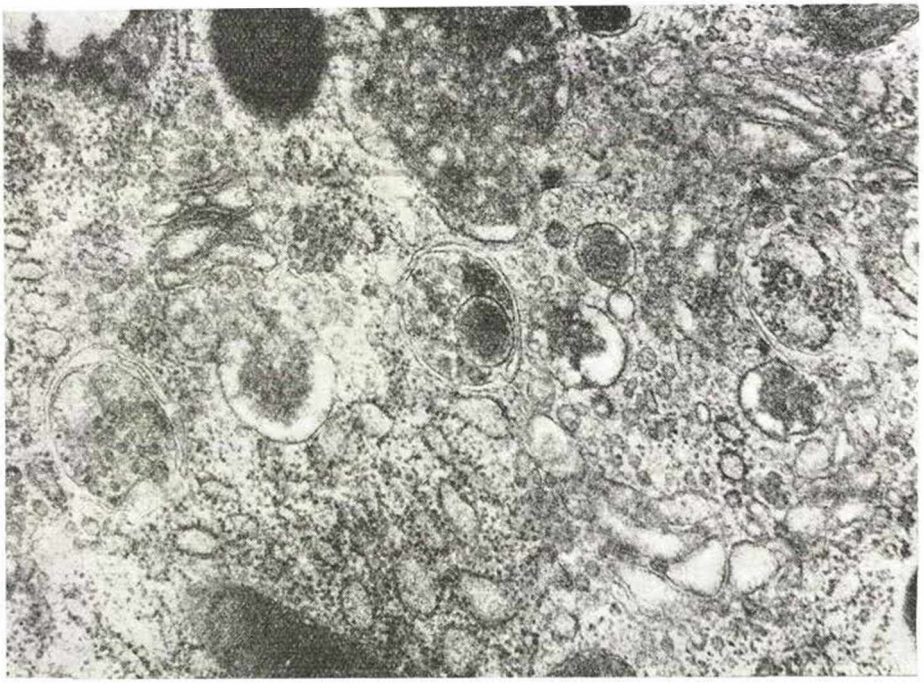

Fig. 10. Autophagic vacuoles containing Golgi vesicles, 3 hours, 15% sucrose solution. (x 38000)

can also be supposed with this type (Figures 3, 4 and 7), at the end of which there can be a vacuole as presented in Figure 6, containing material of low density, as well as vesicles and membranes. However, the number of the intermediate forms is smaller than in the previous case.

The autophagic vacuoles are delimited by a pair (in some instances by several pairs) of membranes, or by one sole membrane. A delimiting pair of membranes is characteristic in the first place for the younger forms which, in their content, present slight degradation. In several cases can it be observed, that the two members of the pair of membranes tightly stick together, cohere or fuse (Figures 3, 8 and 9). Relying on their micrographs, the authors are of the opinion that in pancreatic aci-

Fig. 11. 15% sucrose solution, 3 hours. Gömöri's acid phosphatase reaction. An autophagic vacuole containing no acid phosphatase is shown in the vicinity of a lysosome. (x 26300)

Fig. 12. The same animal, as in Figure 15. Acid-phosphatase positive autophagic vacuole near the Golgi cisternae. (x 29600)

Fig. 13. 15% sucrose solution, 3 hours. Acid-phosphatase positive autophagic vacuoles near the Golgi cisternae. (x 25200)

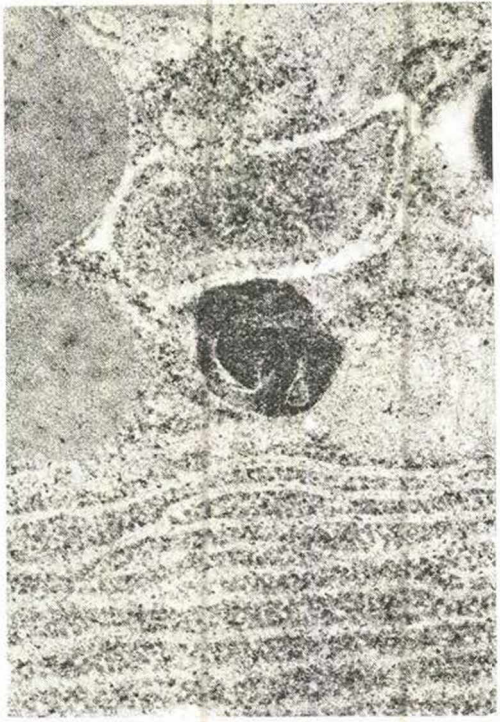

Fig. 14. 15% sucrose solution, 3 hours. A body of high acid -phosphatase activity (probably an older autophagic vacuole) and an acid-phosphatase negative autophagic vacuole can be seen in close contact with one another. (x 34100)

nar cells the membrane of the autophagic vacuoles derives from the cisterns of the ER (Figure 8), or is formed of the membrane system of the Golgi apparatus (Figure 10).

Smooth-surfaced or coated vesicles can often be found near the vacuoles. Frequently the outer member of the delimiting pair of membranes is in connection with the membrane of the smooth-surfaced vesicle fusing with it (Figures 7 and 9).

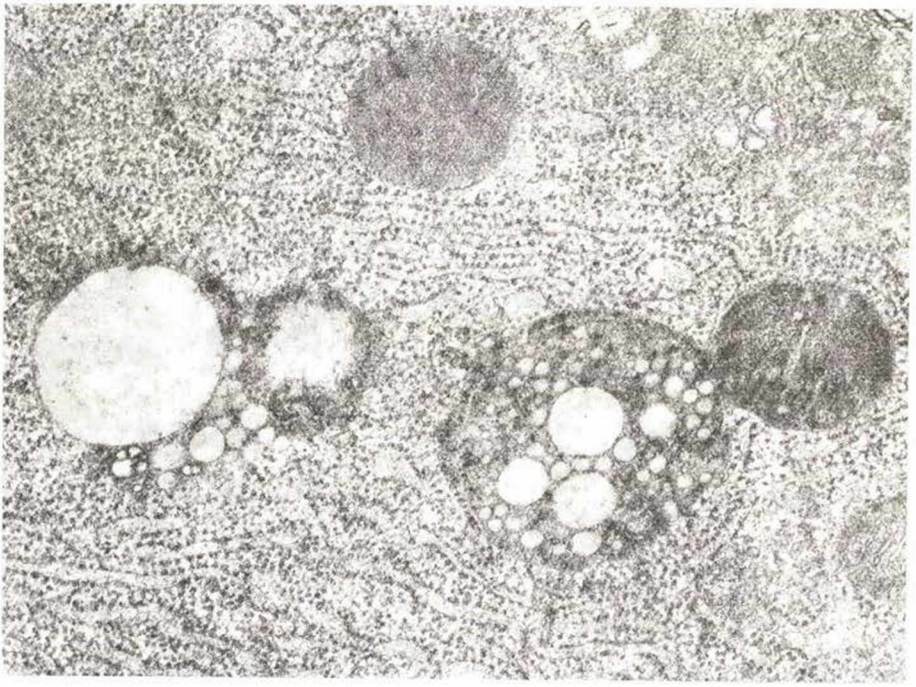

Fig. 15. Cadmium chloride, 2 hours. Vacuolized dense bodies among the cisternae of the ER. (x 25600)

The acid phosphatase activity of the autophagic vacuoles, demonstrable with G ö m ö r i' s method, is variable. Younger vacoules display no activity, however, in the course of degradation it appears, and gradually increases (Figures 11, 12, 13 and 14).

2. A great number of highly vacuolized dense bodies appear among the cisternae of the ER. These contain eletro-lucent vacuoles and vesicles of various size, as well as osmiophilic ground substance, granules, tubuli, and myelin like figures (Figure 15). They show acid phosphatase activity localized to their dense substance (Figure 16). They may be residual bodies, however, the authors did not find any intermediate forms indicative to an origin trough degradation of some other cytoplasmic component.

3. Following both sucrose- and cadmium treatment, a vesicular transformation of the rough surfaced ER can be observed in the basal cytoplasm (Figure 17). In some cases ER cisternae appear in the cytoplasm, the membranes of which have lost their ribosomes (Figure 18).

Along the basal cytoplasm the authors observed multivesicular bodies and numerous small vesicles indicative of pinocytosis.

Fig. 16. 15% sucrose solution, 3 hours. Gömöri reaction. Vacuolized dense bodies near the Golgi cisternae. Acid -phosphatase activity can be seen in their ground substance, as well as in the Golgi cisternae. (x 20000)

Discussion

According to the data of the authors, two morphologically separable types of autophagic vacuoles can be found in damaged pancreatic cells. Characteristic of one of these is, that the density of the segregated substance is higher than that of the surrounding cytoplasm. With increasing osmiophilia, the autophagic vacuoles of this type can be arranged in a progressive series, in which a great number of intermediate forms can be distinguished. This morphological type probable represents a type of autophagy characterized by a low speed of the lytic processes.

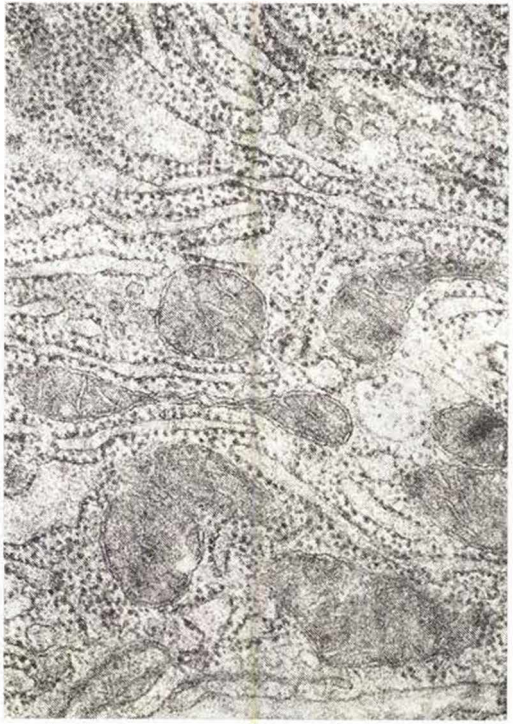

Fig. 17. 15% sucrose solution, 30 minutes. Vesicle groups forming from rough-surface cisternae in the basal cytoplasm. (x 22000)

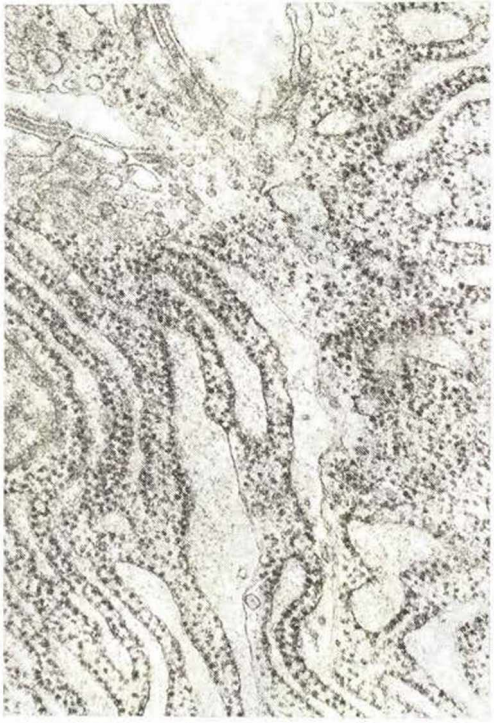

Fig. 18. 15% sucrose solution, 10 minutes. The ER cisternae are in places free from ribosomes. (x 24400)

Of the other type it is characteristic, that the autophagocytized substance shows signs of degradation, however, without a significant increase of osmophilia. In the segregated cisterns changes can be observed even previously to the fusion of the delimiting pair of membranes. The fact, that in that case the segregated cisterns divide into vesicles, and lose their ribosomes indicates a difference in the internal milieu of the vacuole and may be in the permeability of the delimiting membrane. Within this type of autophagic vacuoles, the authors similarly set up a progressive series, with which, however, much less intermediate forms can be ranked than with the first type. Therefore, the authors believe this type of autophagy to be characterized by a relatively high speed of the lytic processes. The two types probably differ from one another first of all in the enzyme content of the vacuoles. This may be indicated by the existence of autophagic vacuoles showing differences in their enzyme content, or not containing demonstrable acid phospatase at all. This results support Arstila and Trump's cell fractionation data (Arstila and Trump 1968), who have demonstrated that as to enzyme content there existed different autophagic vacuoles. The acid-phosphatase positive autophagic vacuoles can be found most frequently near the acid-phosphatase positive cisternae of the Golgi apparatus. Therefore it is likely, that the acid hydrolases are transported into the vacuole by primary lysosomes (Golgi vesicles), or by a secondary one already containing the enzymes (Arstila and Trump, 1968; Ericsson, 1969). The enzyme content may derive from the elements of the autophagocytized Golgi apparatus, or also from the delimiting membrane, if that one is of Golgi origin (Frank and Christensen, 1968).

The autophagic vacuoles of the probable two types are also demonstrable in the pancreatic cells upon neutral-red treatment (Réz and Kovács, 1971). The authors have described similar vacuoles also in the damaged epithelial cells of the seminal vesicle (Kovács and Réz, 1970).

Regarding the origin of the delimiting membrane of the autophagic vacuoles, the authors' findings support the data recently reviewed by Ericsson (Ericsson 1969; Maunsbach, 1969), that it is not a result of *de novo* synthesis. In the authors' experience, in exocrine pancreatic cells, where under normal circumstances neither smooth surfaced ER nor typical GERL can be found, the delimiting membrane of the autophagic vacuole can develop in two ways:

1. A cistern of the rough surfaced ER encircles a part of the cytoplasm, and at the two end it closes by self-fusion thus forming an envelop around the segregated organelles. In the course of the process, the cistern loses its ribosomes. Autophagic vacuoles of that stage can be seen but infrequently, from which it can be presumed that the ribosomes are being lost in an instantaneous manner. The fusion of the delimiting pair of membranes is possibly a slow process; older vacuoles, of which the membrane is but partly fused, are rather frequent. Essner's data refer

the changes taking place in the course of the fusion (Essner 1970), — by applying a modified Kopsch—Kolatschef technique, he demonstrated that, similarly to the Golgi apparatus, the membrane of certain autophagic vacuoles could be impregnated by osmium.

2. In other instances the delimiting pair of membranes is formed by one of the cisternae of the Golgi apparatus. This may be indicated by autophagocytosis of the elements of the Golgi apparatus, as well as by the circumstance that the membrane of the smooth-surfaced vesicles often fuses with the outer member of the delimiting pair of membranes.

A vesicular transformation of rough-surfaced ER also occurs in the epithelial cells of the seminal vesicle (Kovács, 1971). In the acinar cells of the pancreas Hruban found a similar change in the basal cytoplasm, following treatment with azaserine (Hruban, Swift and Slesers, 1965).

As to the origin of the vacuolized dense bodies appearing in great quantity upon treatment with sucrose, cadmium chloride and neutral red (Réz and Kovács, 1969), there are no data at disposal. Theys contain acid phosphatase and, as enzyme donors, play a role in autophagy. Bodies of similar structure, containing melanoid pigments were described in Chédiak—Higashi syndrome (Lutzner, Tierney and Benditt, 1965). They may represent residual bodies of autophagocytosis.

Summary

Hyperosmotic sucrose solution, as well as cadmium ions cause a focal degradation of the cytoplasm of exocrine pancreatic cells, which is manifested by the appearance of large numbers of autophagic vacuoles containing ER fragments, and large-sized, highly vacuolized dense bodies. A vesicular transformation of the rough-surfaced ER and the appearance of smooth-surfaced cisternae were also observed. In the autophagic vacuoles containing intact cisternae there is no demonstrable acid phosphatase activity. On the other hand, a marked enzyme activity can be found in the vacuoles showing degradation.

The membrane of the autophagic vacuoles originates from the membranes of the ER or of the Golgi apparatus. Founded on the morphology of the segregated material, the vacuoles can be classed with two groups. Of the first type it is characteristic that in the course of degradation, the osmiophilia of the segregated substance increases, however, the arrangement of the cisternae remains substantially unchanged. The second type is characterized by a fragmentation and a vesiculation of the encapsuled cisternae, however, at the same time no increase in osmiophilia appears. The authors presume that the deviation of the two types is caused by their different enzyme content.

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